

# The Prevalence of Carbon-13 in Respiratory Carbon Dioxide As an Indicator of the Type of Endogenous Substrate

*The change from lipid to carbohydrate during the  
respiratory rise in potato slices*

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**ABSTRACT** Isotope discrimination is a common feature of biosynthesis in nature, with the result that different classes of carbon compounds frequently display different  $^{13}\text{C}/^{12}\text{C}$  ratios. The  $^{13}\text{C}/^{12}\text{C}$  ratio of lipid in potato tuber tissue is considerably lower than that for starch or protein. We have collected respiratory  $\text{CO}_2$  from potato discs in successive periods through 24 hr from the time of cutting—an interval in which the respiration rate rises 3–5-fold. The  $^{13}\text{C}/^{12}\text{C}$  ratio of the evolved  $\text{CO}_2$  was determined for each period, and compared with the  $^{13}\text{C}/^{12}\text{C}$  ratios of the major tissue metabolites. In the first hours the carbon isotope ratio of the  $\text{CO}_2$  matches that of lipid. With time, the ratio approaches that typical of starch or protein. An estimation has been made of the contribution of lipid and carbohydrate to the total respiration at each juncture. In connection with additional observations, it was deduced that the basal, or initial, respiration represents lipid metabolism—possibly the  $\alpha$ -oxidation of long chain fatty acids—while the developed respiration represents conventional tricarboxylic acid cycle oxidation of the products of carbohydrate glycolysis. The true isotopic composition of the respiratory  $\text{CO}_2$  may be obscured by fractionation attending the refixation of  $\text{CO}_2$  during respiration, and by  $\text{CO}_2$  arising from dissolved  $\text{CO}_2$  and bicarbonate preexisting in the tuber. Means are described for coping with both pitfalls.

## INTRODUCTION

Plant tissues are frequently characterized by a reserve of substrate, a condition which is particularly true for a classical object of plant respiration

study, the storage organ slice. Such tissues do not respond markedly to exogenous substrate. Reserve metabolites are frequently of more than one kind, and it is a matter of some interest to identify the actual endogenous substrate. Respiration in plant tissues may comprise more than one respiratory path. The matter is further complicated by evidence that the relative contribution of coexisting respiratory paths may change with time, as is the case for the sigmoidally increasing respiratory activity of potato discs aerated for 24 hr (1). One piece of evidence pointing to changes in respiratory quality is the concomitant development of malonate sensitivity and cyanide resistance during the aging period (2-4). In addition, aged tissue readily oxidizes glucose and Krebs cycle acids, as measured by  $^{14}\text{CO}_2$  release from exogenously provided labeled compounds, while fresh tissue totally lacks this ability (5). To date the only intimation of the possible nature of the carbon path comprising the basal respiration has been the evolution of  $^{14}\text{CO}_2$  by fresh slices from long-chain, carboxyl-labeled fatty acids. The characteristics of the latter degradation point to  $\alpha$ -oxidation (6). Since the ability to degrade an exogenous carbon source does not necessarily reveal the nature of the endogenous respiratory substrate, we have sought to identify the endogenous substrate by comparing the  $^{13}\text{C}/^{12}\text{C}$  ratio in the normal respiratory  $\text{CO}_2$  with the  $^{13}\text{C}/^{12}\text{C}$  ratio in the various classes of endogenous compounds. The analysis is based on the fact that various classes of tissue metabolites are characterized by their natural relative  $^{13}\text{C}/^{12}\text{C}$  ratios.

Different classes of substrates (e.g. amino acids and lipids) vary from one another in  $^{13}\text{C}/^{12}\text{C}$  ratio as a result of isotopic fractionation during biosynthesis. Extensive fractionation by plant tissue occurs in photosynthetic fixation of carbon dioxide (7), and in dark fixation as well (see below). Such fractionation is indicated by the markedly larger  $^{13}\text{C}/^{12}\text{C}$  ratio of atmospheric  $\text{CO}_2$  than of the total plant carbon (7, 8). To a lesser degree, carbon isotope fractionation occurs in the conversion of carbohydrate to lipid (9, 10), and lesser still during the synthesis of cell wall material (10) and amino acids (9). Altogether, isotopic fractionation during biosynthesis results in different  $^{13}\text{C}/^{12}\text{C}$  ratios in the various classes of metabolites. It is these differences which permit us to determine which substrate is utilized by respiring potato discs during any stage of disc aging.

The procedure first requires determination of the  $^{13}\text{C}/^{12}\text{C}$  ratios of the classes of endogenous metabolites which are potential substrates for potato tuber tissue respiration. As respiration of slices proceeds over a 24 hr period,  $\text{CO}_2$  is periodically collected, and the  $^{13}\text{C}/^{12}\text{C}$  ratio therein is determined. The metabolite which most nearly resembles the respiratory  $\text{CO}_2$  in  $^{13}\text{C}/^{12}\text{C}$  ratio at a given time is taken to represent the predominant respiratory substrate at that time.

Though there have been suggestions regarding the nature of the respira-

tory substrates in aging potato slices from labeling and inhibitor studies (1, 3, 4), direct evidence is lacking. On the basis of a comparison of the  $^{13}\text{C}/^{12}\text{C}$  ratios of respiratory  $\text{CO}_2$  through a 24 hr aging period with the ratios of endogenous metabolites, we have been able to deduce that the respiratory substrate is initially lipid and gives way to starch upon aging.

#### MATERIALS AND METHODS

Tissue discs 1.1 mm thick and 9 mm in diameter were prepared from Idaho Russet Burbank potatoes. Halved tubers were pierced within the vascular ring with a stainless steel cork borer, the core remaining in the tuber. The tuber halves were then sliced with a microtome blade fixed in an appropriate bed (11) to yield discs. At least 100 g of discs washed with 12 liters of  $\text{CO}_2$ -free water were readied for use in 30 min. Slices were prepared at  $2^\circ\text{C}$ .

After slicing, 50–75 g of potato discs were aged in a 1 liter flask containing 200 ml of  $\text{CO}_2$ -free  $10^{-4}\text{ M CaSO}_4$ . The bathing solution was changed four or five times during the 24 hr aging period. The pH of the solution was maintained at the desired value with an automatic titrator. The electrodes and titrant delivery tube were inserted into the bathing solution through a rubber stopper held in a hole in the side of the incubation flask. A pH of 5.0 was maintained with  $0.4\text{ N H}_2\text{SO}_4$ , and a pH of 7.2 with  $0.4\text{ M Tris}$ .

A rubber stopper in the top of the incubation flask held the air inlet and outlet tubes. The outlet tube led to a liquid nitrogen trap, where  $\text{CO}_2$  evolved from the tissue was collected (7). Air entering the flask was scrubbed free of  $\text{CO}_2$  by passage through two 3 ft columns, the first containing Drierite, a desiccant, and the second Ascarite, a  $\text{CO}_2$  absorbent. The flow of air through the system was 110 ml/min.

*Extraction Procedures* Though starch is the predominant food reserve in potato tuber, possible respiratory substrates such as lipids and protein were also isolated and analyzed for their  $^{13}\text{C}/^{12}\text{C}$  ratios. Starch was extracted either by centrifugation of starch granules from a slurry of homogenized potato tuber or by formation of an aqueous starch gel. Starch was first gelled by extracting the tissue with hot 80% ethanol. The starch-containing residue was then boiled in water and centrifuged. The supernatant contained aqueous starch gel. The two methods yielded starch with identical  $^{13}\text{C}/^{12}\text{C}$  ratios, indicating there was no isotopic carbon exchange between starch and hot alcohol. Lipid was extracted from air-dried potato tissue with chloroform-methanol (2:1, v/v). Nonlipid material was extracted from the lipid-containing solvent with  $0.1\text{ M KCl}$ . Protein was prepared by homogenizing tissue in 2%  $\text{NaCl}$ . After centrifugation for 15 min at 10,000 *g*, protein was precipitated by boiling the supernatant for 30 min. The precipitate was dried by lyophilization.

Other types of compounds, e.g. organic acids, present in small amounts in potato, were isolated by ion exchange chromatography as described by Romberger and Norton (4). Compounds adsorbed by the cation and anion exchange resins, respectively, are amino acids and organic acids. The neutral fraction which passes through both resins is composed primarily of sugars. Amberlite or Dowex as exchange resins yielded similar results. All extracted substrates were converted to  $\text{CO}_2$  for mass spectrometry (8).

*Mass Spectrometer Analysis* The  $^{13}\text{C}/^{12}\text{C}$  ratio in any given sample was compared with a standard. The standard used was the  $\text{CO}_2$  from the fossil carbonate skeleton of *Belemnitella americana* (PDB<sub>1</sub>). The function defining the values reported here is

$$\delta^{13}\text{C per mil} = \frac{(^{13}\text{C}/^{12}\text{C}) \text{ sample} - (^{13}\text{C}/^{12}\text{C}) \text{ standard}}{(^{13}\text{C}/^{12}\text{C}) \text{ standard}} \times 1000$$

Thus, a sample with a  $\delta^{13}\text{C}$  per mil of  $-10$  has a  $^{13}\text{C}/^{12}\text{C}$  ratio less than the standard by 10 per mil, or 1.0%. For example, the  $^{13}\text{C}/^{12}\text{C}$  ratio of atmospheric  $\text{CO}_2$  is smaller than that of the PDB<sub>1</sub> standard by about 7 per mil. Therefore, the  $\delta^{13}\text{C}$  per mil of this  $\text{CO}_2$  is  $-7$ . The precision of measuring  $\delta^{13}\text{C}$  with the mass spectrometer is  $\pm 0.1$  per mil.

TABLE I  
 $\delta^{13}\text{C}$  PER MIL OF PRIMARY ENDOGENOUS SUBSTRATES  
FROM POTATO TUBER TISSUE

Substrate	$\delta^{13}\text{C}$ per mil*
Starch	$-25.5$
Protein	$-26.6$
Lipid	$-34.8$

\* Average of four separate determinations.

## RESULTS AND DISCUSSION

The results which follow show that it is possible to determine experimentally whether the endogenous respiratory substrate in potato tuber discs is either lipid or starch and protein. Comparison of the  $^{13}\text{C}/^{12}\text{C}$  ratio of the evolved  $\text{CO}_2$  with that of endogenous tuber metabolites indicates that lipids initially represent the main source of respiratory substrate in fresh tissue discs. Based on our results and previous investigations, it can be deduced that starch becomes the predominant respiratory substrate as aging proceeds. The determination of respiratory substrate during the course of slice aging on the basis of the  $^{13}\text{C}/^{12}\text{C}$  ratio of respiratory  $\text{CO}_2$  proved possible only because the  $^{13}\text{C}/^{12}\text{C}$  ratio of lipid is widely different from other endogenous metabolites as a consequence of carbon isotope fractionation during biosynthesis. The effect of fractionation during respiration upon substrate determination will be discussed rigorously in a later section. For the present, it is enough to indicate that carbon isotope fractionation accompanying respiration is relatively inconsequential, and has little effect on the basic question of whether the predominant substrate utilized by aerated potato discs is lipid or starch.

### $^{13}\text{C}/^{12}\text{C}$ Ratio of Metabolites from Potato

The  $^{13}\text{C}/^{12}\text{C}$  ratios of the common macromolecular components of potato tissue are set out in Table I. Lipid was found to have the most negative  $\delta^{13}\text{C}$

value; i.e. lipid contains less  $^{13}\text{C}$  than protein, which in turn contains less  $^{13}\text{C}$  than starch. The low  $^{13}\text{C}/^{12}\text{C}$  ratio of the lipid fraction relative to other cellular constituents was previously demonstrated in other higher plants, as well as in various algae (9, 10).

It is interesting to note that when lipids are further fractionated by saponification, the nonsaponifiable fraction (mainly isoprenoids) is on the average

TABLE II  
 $\delta^{13}\text{C}$  PER MIL OF ENDOGENOUS INTERMEDIATE  
SUBSTRATES FROM POTATO TUBER TISSUE

Substrates	$\delta^{13}\text{C}$ per mil*
Neutral compounds (sugars)	-23.8
Organic anions (organic acids)	-26.9
Organic cations (amino acids)	-27.1

\* Average of three separate determinations.

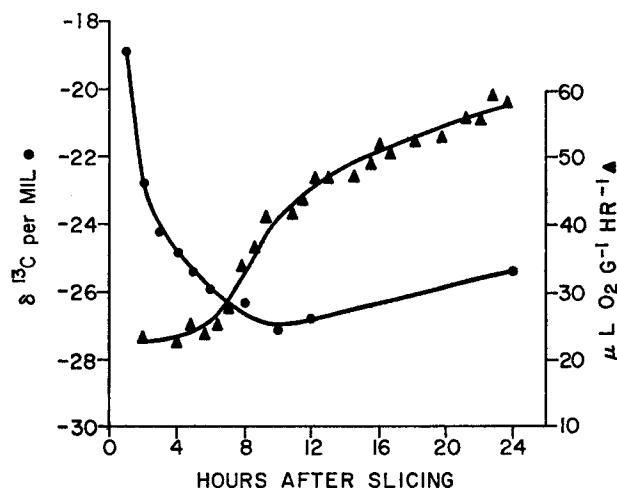


FIGURE 1.  $\delta^{13}\text{C}$  per mil of  $\text{CO}_2$  collected from potato discs in  $\text{CaSO}_4$  solution or a moist atmosphere. The respiratory rate was measured with a Beckman  $\text{O}_2$  analyzer. ●,  $\delta^{13}\text{C}$  per mil; ▲, respiratory rate.

2 per mil heavier than the saponifiable fraction (fatty acids). The difference in  $\delta^{13}\text{C}$  values between the two fractions suggests that  $^{13}\text{C}$ -enriched acetate units are utilized more selectively for the synthesis of malonyl coenzyme A than for acetate condensation reactions.

The  $^{13}\text{C}/^{12}\text{C}$  ratios of compounds considered to be metabolic intermediates are listed in Table II. While the  $\delta$ -values of the major primary components differ from one another (Table I), metabolites derived therefrom closely resemble their macromolecular counterparts. That is, amino acids resemble

protein, and sugars most resemble starch, with respect to their respective  $^{13}\text{C}/^{12}\text{C}$  ratios (Tables I and II).

*Change of Predominant Respiratory Substrate during Aging*

When potato discs were aged either in  $\text{CaSO}_4$  solution or in a moist chamber, the  $\delta^{13}\text{C}$  values of the collected  $\text{CO}_2$ , which at first were high, decreased rapidly during the first 10 hr and rose slightly during the next 16 hr (Fig. 1). While comparison of the  $\delta^{13}\text{C}$  per mil of the collected  $\text{CO}_2$  (Fig. 1) with ap-

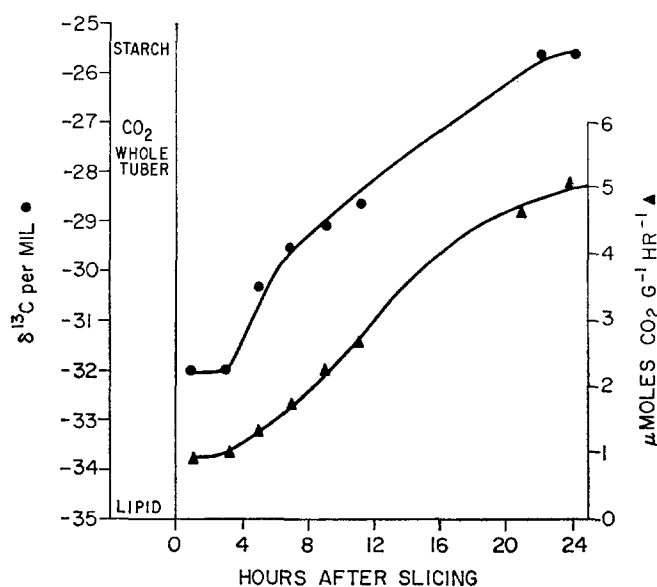


FIGURE 2.  $\delta^{13}\text{C}$  per mil of  $\text{CO}_2$  collected from aging potato discs. The pH of the  $\text{CaSO}_4$  medium was maintained at 5.0 with  $\text{H}_2\text{SO}_4$ . The  $\text{CO}_2$   $\delta$ -values for 1 and 3 hr after slicing were determined from Fig. 6. The column at the left indicates the  $\delta$ -values of the probable primary endogenous substrates and of the respiratory  $\text{CO}_2$  from intact tubers. The respiratory rate represents the  $\text{CO}_2$  collected from discs for 1 hr at the times indicated.

propriate substrate  $\delta$ -values (Table I) clearly indicates that starch or protein is the respiratory metabolite in 24 hr old potato discs, determination of the respiratory substrate during the *early* hours of aging (Fig. 1) is obfuscated by two anomalies. The high  $\delta$ -values of the  $\text{CO}_2$  collected during the initial hours of aging do not resemble any of the known  $\delta$ -values of endogenous substrates (Table I). Furthermore, the *fall* in the  $\delta$ -values (away from starch) observed in the interval from 4 to 10 hr is inconsistent with other indirect evidence which points to the *development* of starch metabolism (via the tri-carboxylic acid cycle) during this period (5).

Two factors distinct from the respiratory process per se have been shown to contribute significantly to the anomalous results of Fig. 1. Both lead to an

enrichment of  $^{13}\text{C}$  in the  $\text{CO}_2$  collected in the first 0–8 hr. When these factors are experimentally obviated, a totally different trend of the  $^{13}\text{C}/^{12}\text{C}$  ratio with time is noted, as depicted in Fig. 2. One cause of the spuriously heavy  $\text{CO}_2$  released in the first hours is the refixation, by carboxylation reactions, of a fraction of the respired  $\text{CO}_2$ . Incorporation probably favors the lighter carbon isotope, and consequently decreases the relative amount of  $^{12}\text{C}$  in the  $\text{CO}_2$  subsequently collected. The second factor that leads to an enrichment of  $^{13}\text{C}$  in  $\text{CO}_2$  evolved by fresh discs is the release of heavy  $\text{CO}_2$  from tissue bicarbonate. Tissue bicarbonate was shown to have a higher  $\delta^{13}\text{C}$  per mil than any other tissue carbon, and the contribution of bicarbonate to respiratory  $\text{CO}_2$  would therefore increase the  $\delta$ -value of the collected  $\text{CO}_2$ . The bicarbonate in question is in the tuber tissue at the time of slicing, not the product of slice respiration following cutting. Evidence of the release of heavy carbon from dissolved bicarbonate is to be found in the transient relatively high  $^{13}\text{C}/^{12}\text{C}$  values of  $\text{CO}_2$  from tomato leaves respiring in the dark following a prolonged period of photosynthesis (7).

Once the effects of  $\text{CO}_2$  refixation and of bicarbonate-derived  $\text{CO}_2$  are taken into account, it is readily apparent that the change in  $\delta$ -values of  $\text{CO}_2$  collected from tissue during the initial hours of aging is misleading and does not reflect substrate utilization (Fig. 1). When the influence of the extraneous factors noted above is either precluded or corrected for, a totally different trend is noted for  $\text{CO}_2$   $\delta$ -values with time after slicing (compare Fig. 1 with Fig. 2). Based on the  $\delta^{13}\text{C}$  values of Fig. 2, the respiratory substrate changes as potato discs age. Assuming for the moment that the corrections which have been made are valid, a comparison of  $\text{CO}_2$   $\delta$ -values at any given time with values of specific classes of substrates indicates that lipid is the predominant metabolite for fresh tissue respiration, while  $\text{CO}_2$  with a  $\delta$ -value resembling starch or protein characterizes the respiration of aged tissue (Fig. 2 and Table I).

Whether starch or protein is the predominant respiratory substrate of aged discs cannot be discerned from the  $\delta$ -values alone. However, a choice between the two substrates is possible in the light of previous investigations. The latter have shown that as the respiratory rate of potato discs increases 4–5-fold during 24 hr of aging, there is an inordinately large increase in  $^{14}\text{CO}_2$  evolution from exogenously provided labeled glucose, concurrent with a similar increase of tricarboxylic acid cycle activity (5, 12). Furthermore, a meticulous balance sheet of potato disc metabolites in relation to time following slicing has shown that extensive protein synthesis during aging occurs primarily at the expense of soluble amino acids and amides (13, 14). The major part of the  $\text{CO}_2$  evolved during aging is accounted for, moreover, by a depletion of starch (13, 14). It thus appears that as discs are aerated over 24 hr, starch becomes the predominant respiratory substrate.

Knowing that the choice of respiratory substrates for aging discs is probably either starch or lipid, we may calculate the relative contribution of  $\text{CO}_2$  from each substrate consistent with the observed  $\delta$ -values of the total respired  $\text{CO}_2$ . Such calculations indicate that 70% of the  $\text{CO}_2$  collected from discs during the initial hours of aging is derived from lipid (Fig. 3). As the time of

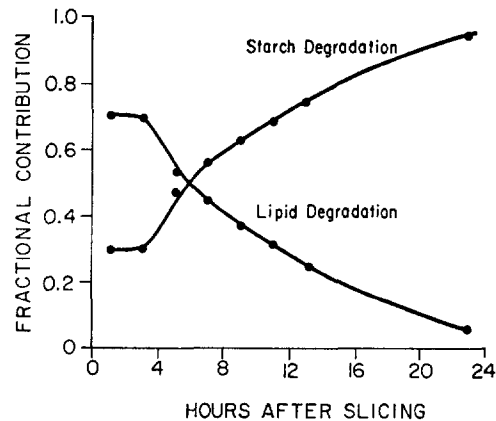


FIGURE 3. The fractional contribution of respiratory  $\text{CO}_2$  from starch and lipid degradation in aging potato discs. The values were calculated from results depicted in Fig. 2. (See the text for selection of starch and lipid as substrates.)

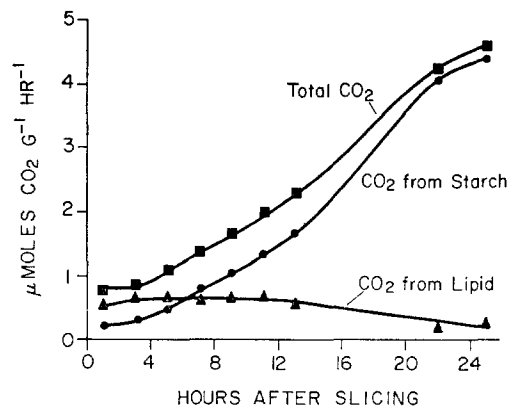


FIGURE 4. The rate of  $\text{CO}_2$  evolution from starch and lipid as a function of potato disc age. Calculated from Figs. 2 and 3. (See the text for selection of starch and lipid as substrates.)

incubation increases, the relative contribution from lipid sharply declines while that from starch increases.

It has been recognized for some time that the respiration of aged tissue is qualitatively distinct from the initial, or basal, respiration (1). As aging progresses, the basal respiration rises but little. The respiratory increment is due primarily to the initiation or activation of another oxidative carbon path. From the fractional contributions from lipid and starch to the  $\text{CO}_2$  throughout the aging process (Fig. 3), we can calculate the actual amount of  $\text{CO}_2$  that should arise from each substrate during the rise in respiration rate (Fig. 4). These calculations suggest that lipid is the primary substrate for



basal respiration, since the rate of  $\text{CO}_2$  production from lipid oxidation remains relatively constant for 12 hr. The increase of respiratory  $\text{CO}_2$  evolution with time is most likely related to the ever-increasing utilization of starch, and the developed respiration is again distinguished from the basal respiration.

*The Basis of Anomalously Heavy  $\text{CO}_2$  and Correction Therefor*

**$\text{CO}_2$  REFIXATION** Since  $\text{CO}_2$  fixation is generally accompanied by a significant selective utilization of  $^{12}\text{C}$  (7), the refixation of respired  $\text{CO}_2$  by potato discs would be expected to raise the  $\delta^{13}\text{C}$  of evolved  $\text{CO}_2$  over that arising directly from respiration. It appears that bicarbonate rather than  $\text{CO}_2$  per se is involved in dark  $\text{CO}_2$  fixation in higher plant tissue. Fixation increases sharply with rising external pH.<sup>1</sup> The extent of  $^{13}\text{C}$  enrichment may be expected to be proportional to the amount of refixation, and to the extent of fractionation favoring  $^{12}\text{C}$  which occurs during carboxylation.

To assess whether  $\text{CO}_2$  refixation in fact increases the  $\delta^{13}\text{C}$  values of respiratory  $\text{CO}_2$ , potato tissue was incubated at either pH 5.0 or 7.2, and the  $\delta$ -values of the  $\text{CO}_2$  trapped were determined at hourly intervals. It is important to emphasize that  $\delta$ -measurements were made on the *total*  $\text{CO}_2$  produced in the designated period. That is, for experiments at pH 7.2, the bathing solution of a separate sample was acidified at the end of each interval, and the  $\text{CO}_2$  so released from bicarbonate was collected and combined with the  $\text{CO}_2$  trapped from the air stream during the same interval.

The effect of pH on the  $\delta^{13}\text{C}$  per mil of  $\text{CO}_2$  collected from potato discs aged for 24 hr is depicted in Fig. 5. The curves show that the  $\delta$ -values of the respired  $\text{CO}_2$  are at all times more negative at pH 5.0. The difference in  $\delta$ -value between  $\text{CO}_2$  collected at pH 7.2 and  $\text{CO}_2$  collected at pH 5.0 is greatest after 3 hr of aging and subsequently diminishes with time. If the isotope fractionation attending dark  $\text{CO}_2$  fixation in our experiments is similar to that attending the carboxylation of ribulose diphosphate (7), the higher  $\delta$ -value of  $\text{CO}_2$  collected at pH 7.2 should be due to greater  $\text{CO}_2$  refixation, and the amount of  $\text{CO}_2$  collected at pH 7.2 (after acidification) should be less than that collected at pH 5.0. Furthermore, one should be able to calculate the amount of  $\text{CO}_2$  that must be refixed in order to get the differences in  $\delta$ -values observed in Fig. 5.

The calculation is simplified by the knowledge that the organic acids synthesized by nonphotosynthetic  $\text{CO}_2$  fixation are sequestered in the vacuole and not readily respired (16, 17).<sup>1</sup> If we know the  $\delta$ -values of the total evolved  $\text{CO}_2$ , on the one hand, and of the  $\text{CO}_2$  emitted with concomitant refixation, on the other, we can readily calculate the relative amounts of emitted and refixed  $\text{CO}_2$ . The  $\delta$ -value of the total respiratory  $\text{CO}_2$  is taken to be that of

<sup>1</sup> Jacoby, B., and G. G. Laties. Unpublished observations (see ref. 15).

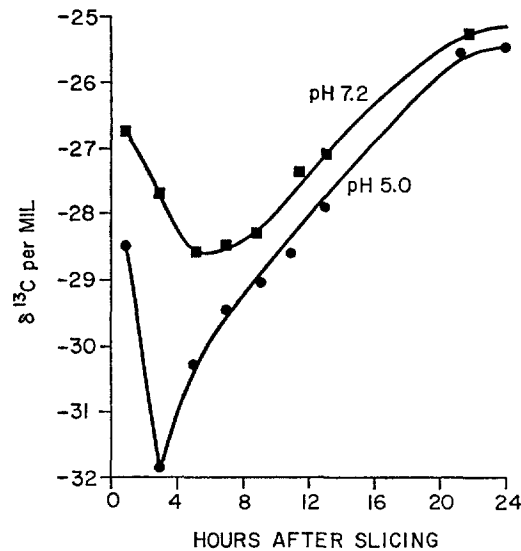


FIGURE 5. The effect of pH on changing the  $\delta^{13}\text{C}$  per mil of  $\text{CO}_2$  collected from potato discs. Discs were incubated at either pH 5.0 (●) or 7.2 (■). At the end of each interval, the bathing solution was acidified with  $\text{H}_3\text{PO}_4$ , and the  $\text{CO}_2$  so released was combined with the  $\text{CO}_2$  trapped during the experimental interval.

the  $\text{CO}_2$  collected at pH 5.0, at which pH little net nonphotosynthetic  $\text{CO}_2$  fixation by plant tissue takes place (18).<sup>1</sup> By contrast, significant refixation occurs at pH 7.2, and the  $\delta$ -value of  $\text{CO}_2$  collected at pH 7.2 (after acid tipping) is influenced by isotopic fractionation during refixation. If we assume that the carbon isotope fractionation during dark fixation is the same as for the carboxylation of ribulose observed in tomato plants (7), we can calculate the  $\delta$ -value for the refixed  $\text{CO}_2$ . The fractionation factor,  $\alpha$ , is described as follows (see ref. 7):

$$\alpha = \frac{{}^{13}\text{C}/{}^{12}\text{C} \text{ total } \text{CO}_2}{{}^{13}\text{C}/{}^{12}\text{C} \text{ fixed } \text{CO}_2} \cong \frac{\delta \text{ total } \text{CO}_2 - \delta \text{ fixed } \text{CO}_2}{1000} + 1 \quad (1)$$

The fractionation factor as determined by Park and Epstein (7) is 1.017; that is, the  $\delta$ -value of the fixed  $\text{CO}_2$  is  $-17$  per mil compared with the  $\delta$ -value of the total  $\text{CO}_2$ .

The conservation equation for the determination of the relative proportion of refixed  $\text{CO}_2$ , ( $x$ ), and emitted  $\text{CO}_2$ , ( $1 - x$ ), is as follows.

$$\delta \text{ total } \text{CO}_2 (\text{pH } 5.0) = (x) \delta \text{ emitted } \text{CO}_2 (\text{pH } 7.2) + (1 - x) \delta \text{ refixed } \text{CO}_2 (\text{pH } 7.2) \quad (2)$$

In consequence

$$\text{Fraction refixed} = \frac{\delta \text{ CO}_2 (\text{pH } 5.0) - \delta \text{ CO}_2 (\text{pH } 7.2)}{1000 (\alpha - 1)} \quad (3)$$

The fraction of  $\text{CO}_2$  refixed at pH 7.2 at the time when the difference in  $\delta$ -values is the greatest (3 hr after slicing, Fig. 5) is 0.24; i.e. 24% of the total respiratory  $\text{CO}_2$  is refixed. The fractional decrease of  $\text{CO}_2$  (determined with a Warburg apparatus) evolved by tissue at pH 7.2 relative to pH 5.0 was 0.36, which indicates a refixation of 36% of the total  $\text{CO}_2$  evolved by respiration. The rate of respiration as measured by oxygen utilization did not differ with pH, indicating that the respiration rate per se is unaffected by the pH of the ambient solution. Thus, since the manometrically observed fractional decrease in emitted  $\text{CO}_2$  is at least as great as the calculated decrease, it is reasonable to conclude that the difference between the  $\delta$ -values of  $\text{CO}_2$  collected at pH 7.2 and pH 5.0 is due largely to  $\text{CO}_2$  refixation.

These data demonstrate that unless the pH is kept low,  $\text{CO}_2$  refixation markedly raises the observed  $\text{CO}_2$   $\delta$ -values and leads to a spuriously high value which does not reflect the nature of the respiratory substrate (Fig. 1).

**RESIDUAL  $\text{CO}_2$**  Even when care is taken to minimize the effects of  $\text{CO}_2$  refixation, the  $\delta$ -value for  $\text{CO}_2$  collected during the first hour remains inordinately high (Fig. 5). As will be seen, the high  $\delta$ -value for the first hour originates in residual  $\text{CO}_2$ -bicarbonate, i.e. in  $\text{CO}_2$  and bicarbonate in solution in the intact tuber and present in the discs at the time of slicing. The partial pressure of  $\text{CO}_2$ , and hence the quantity of dissolved  $\text{CO}_2$  in potato tubers, may be quite high (19).

Residual  $\text{CO}_2$  and  $\text{HCO}_3^-$  are in time released from discs by diffusive efflux and exchange with respired  $\text{CO}_2$ . In consequence the initial  $\delta$ -values of the  $\text{CO}_2$  collected from fresh tissue reflect values for metabolic  $\text{CO}_2$  adulterated by residual  $\text{CO}_2$ . Experiments to be discussed will show that the  $\delta$ -value of the residual  $\text{CO}_2$  and  $\text{HCO}_3^-$  is considerably higher than substrate  $\delta$ -values, and that removal of the residual fraction lowers the  $\delta$ -value of initially evolved  $\text{CO}_2$  toward that of lipid. Furthermore, since the time required to eliminate residual bicarbonate by exchange with respired  $\text{CO}_2$  is decreased by decreasing disc thickness, we were able to determine the  $\delta$ -value of respired  $\text{CO}_2$  without the contribution of the residual fraction.

Residual  $\text{CO}_2$  and  $\text{HCO}_3^-$  were isolated by homogenizing tubers in an air-tight Waring Blendor at  $-4^\circ\text{C}$ . During homogenization, the  $\text{CO}_2$  released was swept into a liquid nitrogen trap by passing a stream of  $\text{CO}_2$ -free air through the blender. The  $\text{CO}_2$  collected during the first 10 min of homogenization was considered primarily residual  $\text{CO}_2$  per se. Later samples were considered to arise from bicarbonate. The latter samples were collected for 10 min in a stream of  $\text{CO}_2$ -free air after each of two consecutive 40 min periods in which the homogenate was constantly stirred. Residual bicarbonate differs in  $\delta$ -value from residual  $\text{CO}_2$  by about  $-7$  per mil, a value in accordance with theoretical expectations (8, 9). The values of the  $\delta^{13}\text{C}$  per mil for  $\text{CO}_2$  and  $\text{HCO}_3^-$  are  $-26.9$  and  $-19.1$ , respectively (Table III).

It is apparent that the  $\delta$ -values of residual  $\text{CO}_2$ , and particularly of residual  $\text{HCO}_3^-$ , are sufficiently large that their contribution to respiratory  $\text{CO}_2$  leads to  $\text{CO}_2$   $\delta$ -values greater than those of the substrates (Fig. 1; cf. Fig. 2). Furthermore, the  $\delta$ -value of  $-19.1$  per mil for residual  $\text{HCO}_3^-$  is sufficient evidence that neither starch nor protein is the predominant substrate for fresh tissue respiration. Since  $\text{CO}_2$  arising from starch or protein will have

TABLE III  
 $\delta^{13}\text{C}$  PER MIL OF  $\text{CO}_2$  AND  $\text{HCO}_3^-$  FROM  
POTATO TUBER TISSUE

Compound determined	Time after homogenizing	Tissue content (fresh weight)	$\delta^{13}\text{C}$ per mil
	min	$\mu\text{moles/g}$	
$\text{CO}_2^*$	10	0.45	$-26.9$
$\text{HCO}_3^- \dagger$	70	1.50	$-18.7$
	130	1.00	$-19.5$

\* Average of three determinations.

† Average of two determinations.

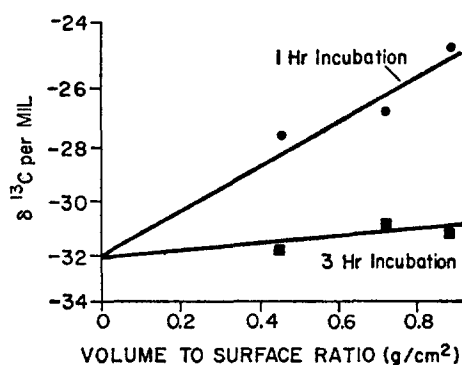


FIGURE 6.  $\delta^{13}\text{C}$  per mil of evolved  $\text{CO}_2$  as a function of disc thickness at constant disc diameter.  $\text{CO}_2$  was collected in the intervals from 0.5 to 1.5 hr (●) and from 2.5 to 3.5 hr (■) after slicing. Incubation conditions were the same as in Fig. 2.

a  $\delta$ -value of about  $-26$  per mil (Table I), any contribution by residual bicarbonate to  $\text{CO}_2$  derived from starch or protein will raise the  $\delta$ -value above  $-26$ . Since the observed value for  $\text{CO}_2$  from fresh discs is  $-28.3$  per mil (Fig. 5; 1 hr after slicing), it is probable that lipid and not starch or protein is the initial (or basal) substrate.

To estimate quantitatively the contribution of lipid oxidation to total fresh disc respiration, we must know the  $\delta$ -value for respiratory  $\text{CO}_2$  unobscured by refixation or by residual  $\text{CO}_2$ -bicarbonate. To this end an estimation was made of the  $\delta$ -value of the respiratory  $\text{CO}_2$  of discs of infinite thinness held at pH 5.0. With but a short preincubation period, it is to be expected that respiratory  $\text{CO}_2$  from very thin discs should be devoid of con-

taminating residual  $\text{CO}_2$ , since the rate of purging of preexisting  $\text{CO}_2$  from tissue slices will be inversely related to the thickness (20). In Fig. 6 the  $\delta$ -value for respiratory  $\text{CO}_2$  at pH 5.0 has been plotted as a function of relative disc thickness (volume to surface ratio). The influence of disc thickness on the  $\delta$ -value is readily perceived, as is the effect of preincubation. The unambiguous  $\delta\text{CO}_2$  value for fresh tissue respiration, as determined from the ordinate intercept in Fig. 6, is  $-32$ , a value typical of lipid. A precise  $\delta$ -value for the respiratory  $\text{CO}_2$  from fresh slices permits a calculation of the quantitative contribution of lipid metabolism to fresh slice respiration (Figs. 3 and 4).

In summary, when the external pH is kept at 5.0, and correction is made for the contribution of residual  $\text{CO}_2$ -bicarbonate to the over-all  $\delta$ -value for respiratory  $\text{CO}_2$ , the  $\delta$ -values of respiratory  $\text{CO}_2$  accurately indicate whether lipid is the initial endogenous substrate and delineate the shift in substrate with time in aging discs. All deductions are based on the presumption that there is little fractionation during substrate degradation, and, as shown below, such appears to be the case.

#### *Carbon Isotope Fractionation during Substrate Degradation*

The difference in carbon  $\delta$ -values of lipids and carbohydrates reflects fractionation during biosynthesis. The question of concern is whether fractionation during the degradation of a *given* substrate is large enough to obscure any difference between substrates of disparate  $\delta$ -values. The answer would appear negative, since when substrate is plentiful, i.e. when the decrease in substrate concentration is negligible during the experimental period, the difference in  $\delta$ -values between the substrate and  $\text{CO}_2$  derived therefrom depends solely upon the degree of fractionation in the first step of a unidirectional sequence. Only at metabolic branch points will there be further fractionation in the steady state. Any deviation of the  $\delta$ -values between  $\text{CO}_2$  and substrate during respiration will depend upon the relative prevalence of synthetic side events which do not lead to complete combustion.

While starch metabolism leads to a variety of metabolic intermediates as well as to  $\text{CO}_2$ , it is noteworthy that all major metabolites other than lipid have a carbon  $\delta$ -value akin to that of starch (Tables I and II). Thus, when starch is the substrate, fractionation even at metabolic branch points would appear to be of no consequence in degradation. However, were starch converted to lipid and  $\text{CO}_2$ , the  $^{13}\text{C}$  enrichment of lipids would be accompanied by a  $^{13}\text{C}$  enrichment of the evolved  $\text{CO}_2$  (10). On the other hand, since it could be inferred from a previous investigation with potato discs aged for 3 hr (4) that the rate of lipid synthesis on a carbon basis is less than 1% of the rate of  $\text{CO}_2$  production, the enrichment of respiratory  $\text{CO}_2$  with  $^{13}\text{CO}_2$  due to lipid synthesis would be negligible. Based on the  $\delta$ -values of potato substrates, as much as 10% conversion of starch to lipid would cause but a 1 per mil increase in the  $\delta$ -value of the respiratory  $\text{CO}_2$  over that of starch.

An estimation of the over-all carbon isotope fractionation during respiratory degradation of a given substrate can be obtained from examination of a totally heterotrophic organism. A growing culture of the fungus *Fusarium roseum* was selected as a test organism, since the anticipated prevalence of synthetic reactions concomitant with respiratory metabolism should place an upper limit to the expected deviation of  $\delta$  between substrate and respiratory  $\text{CO}_2$ .

Cultures of *F. roseum* were grown aerobically on inorganic salts medium (21) with a sole carbon source of either glucose or palmitic acid. During the logarithmic growth phase, respired  $\text{CO}_2$  was collected and analyzed for its  $\delta^{13}\text{C}$  value. These values were then compared with those of the substrates. As indicated in Table IV, the deviations of the  $\delta$ -values of the  $\text{CO}_2$  from those

TABLE IV  
 $\delta^{13}\text{C}$  PER MIL OF SUBSTRATES AND  $\text{CO}_2$  FROM  
AEROBICALLY GROWN *F. ROSEUM*

	$\delta^{13}\text{C}$ per mil*	
	Substrate	$\text{CO}_2$
Glucose	-10.7	-9.4
Palmitic acid	-22.4	-20.5

\* Average of three determinations.

of the corresponding substrates are 1.0 with glucose and 1.9 with palmitic acid. Therefore, the maximum increase of the  $\text{CO}_2$   $\delta$ -value over that of the substrate should be no greater than 1.9 per mil for potato tuber discs.

If the average maximum deviation between  $\delta$ -values of substrate and  $\text{CO}_2$  derived therefrom were as great as 1.5 per mil for any substrate or mixture of substrates utilized, the change from predominantly lipid to predominantly starch oxidation would not be obscured. For example, when the  $\delta$ -value of substrate and  $\text{CO}_2$  derived therefrom are taken to be identical, the respiratory contribution of starch at 24 hr is estimated to be 95% (Fig. 3). With a 1.5 per mil deviation during degradation, the estimated contribution of starch drops to 86%. The decrease from 95 to 86% is inconsequential in evaluating the change from lipid to starch degradation by potato discs aerated for 24 hr.

#### CONCLUSION

There is little question that the respiration which develops with time in storage organ slices is different in kind from the basal, or initial, respiration (1, 22). Evidence presented herein has reaffirmed this judgment, and elucidated one of the basic elements comprising the change in respiratory characteristics, i.e. the transition from lipid to carbohydrate as the predominant

respiratory substrate. In contrast to the rise in respiration which develops with time in thin slices, the instantaneous rise on slicing has, tacitly at least, been considered simply an enhancement of tuber respiration. However, the  $\delta$ -value of the respiratory  $\text{CO}_2$  from intact tubers (Fig. 2) is very close to that of the carbon in starch or protein (Table I), and markedly higher than the  $\delta$ -value of  $\text{CO}_2$  evolved by fresh slices, which appears to arise from lipid.

The respiration rate of freshly cut slices may be from 3–5 times that of the intact tuber on a fresh weight basis (1). From measured respiration rates and  $\text{CO}_2$   $\delta$ -values, we have calculated that the  $\text{CO}_2$  produced from carbohydrate (and perhaps protein) is about  $0.14 \mu\text{mole g}^{-1} \text{hr}^{-1}$  in the intact tuber and  $0.18 \mu\text{mole g}^{-1} \text{hr}^{-1}$  in fresh discs. On the other hand, lipid oxidation increases from less than  $0.04 \mu\text{mole CO}_2 \text{ g}^{-1} \text{hr}^{-1}$  in the tuber to about  $0.6 \mu\text{mole CO}_2 \text{ g}^{-1} \text{hr}^{-1}$  in fresh discs.

The respiratory rise on slicing seems to involve the sudden onset of lipid metabolism. What is the source of lipid? While the lipid content of potato is small [0.13% of the fresh weight (23)], the amount of lipid required to support the calculated rate of lipid oxidation for 25 hr (Fig. 4) would be but 12% of the total lipid present. Jackman and Van Steveninck (24) have shown that the membranous endoplasmic reticulum of beet root virtually disintegrates during the first hours following slicing—later to reappear. Mitochondrial membrane structure is seriously disorganized as well for a limited time (25). Furthermore, the onset of lipase activity has been shown to attend the maceration of seedlings (26). Lipid metabolism *may* reflect the breakdown of cellular membranes, in which case we may have to face the disagreeable task of resurrecting the concept of wound respiration.

Fresh tissue slice respiration is largely cyanide-sensitive and malonate-resistant—characteristics shared by the  $\alpha$ -oxidation of long-chain fatty acids (6, 27, 28). The capacity for  $\alpha$ -oxidation of fatty acids in potato slices remains fairly constant with aging, as does the malonate-resistant basal respiration. It seems at least possible, therefore, that the  $\alpha$ -oxidation of lipids comprises the basal respiration. Cyanide causes a transition from carbohydrate to lipid oxidation in ostensibly cyanide-resistant aged potato slices.<sup>2</sup> In this connection it is interesting that cyanide stimulates the respiration of intact potato tubers (29).

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